BAK1 Is Not a Target of the *Pseudomonas syringae* Effector AvrPto

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Plant cell surface-localized receptor kinases such as FLS2, EFR, and CERK1 play a crucial role in detecting invading pathogenic bacteria. Upon stimulation by bacterium-derived ligands, FLS2 and EFR interact with BAK1, a receptor-like kinase, to activate immune responses. A number of *Pseudomonas syringae* effector proteins are known to block immune responses mediated by these receptors. Previous reports suggested that both FLS2 and BAK1 could be targeted by the *P. syringae* effector AvrPto to inhibit plant defenses. Here, we provide new evidence further supporting that FLS2 but not BAK1 is targeted by AvrPto in plants. The AvrPto-FLS2 interaction prevented the phosphorylation of BIK1, a downstream component of the FLS2 pathway.

The ability of plants to recognize invading pathogens and activate immune responses is critical for plant survival. As the first layer of induced immunity, plants use cell surface-localized pattern-recognition receptors (PRR), typically receptor kinases or receptor-like proteins, to detect conserved microbial molecular signatures termed pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs), thereby activating PAMP-triggered immunity (PTI) (Chisholm et al. 2006; Zipfel 2008, 2009). For example, the *Arabidopsis* receptor kinases FLS2 and EFR are receptors of bacterial flagellar peptide flg22 and elongation factor-Tu peptide elf18, respectively (Chinchilla et al. 2006; Zipfel et al. 2006). Upon exposure to ligands, FLS2 and EFR rapidly associate with another receptor-like kinase, BAK1, which is also known to be required for BRI-mediated brassinosteroid (BR) signaling (Chinchilla et al. 2007; Heese et al. 2007; Schulze et al. 2010). *Arabidopsis* mutants defective in PRR or PTI signaling components are compromised in resistance to pathogens (Gimenez-Ibanez et al. 2009; Miya et al. 2007; Wan et al. 2008; Zipfel et al. 2004, 2006). Conversely, heterologous expression of *Arabidopsis* EFR in solanaceous plants resulted in increased resistance to several bacterial pathogens (Lacombe et al. 2010). More importantly, recent studies showed that PRR and PTI signaling components are frequently targeted by pathogen virulence factors, indicating that PTI is critically important for plant disease resistance (Fu et al. 2007; Gimenez-Ibanez et al. 2009; Gohre et al. 2008; Xiang et al. 2008; Zhang et al. 2007, 2010; Wang et al. 2010).

The *Pseudomonas syringae* type III secretion system delivers approximately 30 effector proteins into the host cell. It is

well-established that many of these effector proteins target important host components to defeat plant immunity (Block et al. 2008; Boller and He 2009; Gohre and Robatzek 2008; Zhang and Zhou 2010; Zhou and Chai 2008). Among these effectors, AvrPto and AvrPtoB have been investigated in greater details. In tomato plants, they both interact with the protein kinase Pto and trigger disease resistance specified by the corresponding resistance protein Prf (Abramovitch et al. 2003; Tang et al. 1996). Prf constitutively interacts with Pto and is inactive in the absence of AvrPto or AvrPtoB (Mucyn et al. 2006). The interaction of Pto with AvrPto or AvrPtoB presumably alters the conformation of the Pto-Prf complex, switching Prf into an active state (Wu et al. 2004; Xing et al. 2007). In susceptible tomato and *Arabidopsis* plants, AvrPto and AvrPtoB are important virulence determinants (Abramovitch et al. 2003; Shan et al. 2000). We showed that AvrPto can interact with both FLS2 and EFR in vitro and in vivo and that this interaction is correlated with the virulence function of AvrPto (Xiang et al. 2008). The functional significance of FLS2-AvrPto interaction was further supported by the restoration of virulence to an *avrPto*-deficient strain of *P. syringae* by an *fls2* mutation in *Arabidopsis* plants. Recent findings from other groups indicate that AvrPtoB also targets PRR (Gimenez-Ibanez et al. 2009; Gohre et al. 2008). The AvrPtoB protein consists of an N-terminal domain and a C-terminal E3 ligase (Abramovitch et al. 2006; Janjusevic et al. 2006), and the E3 ligase activity was shown to mark FLS2 (Gohre et al. 2008) or the putative chitin receptor CERK1 for degradation (Gimenez-Ibanez et al. 2009). Together these studies indicate that AvrPto and AvrPtoB both target PRR to enhance bacterial virulence.

Contrary to the studies described above, Shan and associates (2008) reported that AvrPto interacted with BAK1 more strongly than with FLS2 and suggested that BAK1 but not FLS2 is a physiological target for AvrPto. In this study, we performed additional experiments to thoroughly test the interactions between AvrPto and FLS2, AvrPto and BAK1, and FLS2 and BAK1. The results show that FLS2 but not BAK1 is targeted by AvrPto.

RESULTS

Interaction of AvrPto with FLS2 and BAK1 in protoplasts.

The ligand-induced formation of the FLS2-BAK1 receptor complex enables the activation of downstream signaling (Wang et al. 2008). An interaction of AvrPto with either FLS2 or BAK1 can explain its inhibition of flg22-induced signaling events and defense responses. To determine if AvrPto preferentially interacts with FLS2 or BAK1, we coexpressed FLAG-

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tagged FLS2 and BAK1 along with a hemagglutinin (HA)-tagged AvrPto in *Arabidopsis* protoplasts and performed coimmunoprecipitation (co-IP) assays. Figure 1A shows that both FLS2-FLAG and BAK1-FLAG accumulated to a similar level in protoplasts. Proteins isolated by anti-HA IP contained only FLS2-FLAG but not BAK1-FLAG, supporting the idea that only FLS2 is targeted by AvrPto. It should be noted that, in our experiments, the immunoprecipitated beads were stringently washed to prevent nonspecific interactions. As a result, not even a trace amount of FLS2 signal was seen when IP was performed in the absence of AvrPto. To further verify this result, we constructed HA-tagged FLS2 and BAK1 and coexpressed them with FLAG-tagged AvrPto. Anti-FLAG IP showed that only FLS2 was detected in the AvrPto immune complex (Fig. 1B). To test if a BAK1-AvrPto interaction

could be detected when BAK1 was first immunoprecipitated, we performed anti-HA IP on the protein extract shown in Figure 1B. Again, AvrPto-FLAG was found to associate with FLS2-HA but not BAK1-HA (Supplementary Fig. 1). Thus while the FLS2-AvrPto interaction was reliably detected in co-IP assays, a BAK1-AvrPto interaction was not detected in these experiments.

In some anti-HA co-IP experiments, however, a weak BAK1-FLAG signal was detected under long exposures when BAK1-FLAG, FLS2-FLAG, and AvrPto-HA were coexpressed in the same protoplasts but not in the absence of FLS2-FLAG overexpression (Fig. 1C). This result suggests that overexpression of FLS2 and BAK1 in the same cell might have caused a low level of ligand-independent FLS2-BAK1 interaction that led to an indirect interaction between BAK1 and AvrPto. To further

Fig. 1. Coimmunoprecipitation (co-IP) assays for AvrPto-FLS2 and AvrPto-BAK1 interactions in protoplasts. **A**, FLAG-tagged FLS2 but not BAK1 interacts with hemagglutinin (HA)-tagged AvrPto. The experiment was repeated three times with similar results. **B**, HA-tagged FLS2 but not BAK1 interacts with FLAG-tagged AvrPto. The experiment was repeated six times with similar results. **C**, Flg22 treatment recruits BAK1 into the AvrPto-FLS2 complex. Protoplasts were treated with 1 μ M flg22 for 5 min before co-IP was performed. The experiment was repeated three times with similar results. IP = immunoprecipitation, IB = immunoblot.

test this possibility, we treated these protoplasts with flg22 prior to co-IP. Indeed, the flg22 treatment significantly enhanced the BAK1-FLAG signal in the immune complex (Fig. 1C).

In previous studies (Shan et al. 2008; Xiang et al. 2008), the FLS2-AvrPto and BAK1-AvrPto interactions in the plant cell were examined only with co-IP assays. Because co-IP assays involve multiple steps of sample handling, the procedures may be prone to lab-to-lab variations. This may, in part, explain the inconsistency of results between the two published studies. Therefore, we performed bimolecular fluorescence complementation (BiFC) assays (Walter et al. 2004), which involve

relatively few steps of sample handling. Expression of the BAK1-cYFP (cytosolic yellow fluorescent protein) fusion protein in *bak1* protoplasts stimulated *FRK1-LUC* reporter expression in the presence of flg22 (Supplementary Fig. 2), indicating that our fusion construct was functional. YFP fluorescence was detected only when AvrPto-nYFP (the N-terminal part of YFP) was coexpressed with FLS2-cYFP but not with BAK1-cYFP (Fig. 2), although the protein was expressed at similar levels across different samples. These results further demonstrated that AvrPto is capable of interacting with FLS2 but not BAK1 in plant cells.

Fig. 2. Bimolecular fluorescence complementation assay for FLS2-AvrPto and BAK1-AvrPto interactions. FLS2-cYFP or BAK1-cYFP were cotransfected with AvrPto-nYFP or the SPYNE vector (nYFP) into protoplasts, and the protoplasts were visualized under a confocal microscope. The images shown are representative of multiple protoplasts (FLS2-cYFP+AvrPto-nYFP, 53; BAK1-cYFP+AvrPto-nYFP, 61; FLS2-cYFP+nYFP, 45; BAK1-cYFP+nYFP, 50). Immunoblots (IB) show protein levels as detected with anti-AvrPto (for AvrPto-nYFP) and anti-HA (hemagglutinin) (for FLS2-cYFP and BAK1-cYFP) antibodies. The experiment was repeated twice with similar results.

AvrPto interacts with endogenous FLS2 in plants.

Because protoplast assays may or may not reflect physiological conditions in plants, we tested if AvrPto interacts with endogenous FLS2 in transgenic seedlings carrying an estrogen-inducible *AvrPto-FLAG* transgene. Anti-FLAG co-IP experiments specifically detected FLS2 protein in the *AvrPto*-transgenic seedlings but not nontransgenic seedlings (Fig. 3). Anti-BAK1 immunoblot failed to detect BAK1 in the same immune complex (Fig. 3). These results further support that, in plants, AvrPto interacts with FLS2 but not BAK1.

AvrPto can interact with BSK3 and CDG1.

Transgenic plants overexpressing *AvrPto* were shown to display BR signaling defects resembling *brl1* and *bak1* mutants (Shan et al. 2008). This led to the proposal that BAK1 is targeted by AvrPto, because BAK1 is required for both BR signaling and PAMP signaling. We examined morphological phenotypes of seedlings expressing the *AvrPto* transgene under the estrogen-inducible promoter. The estradiol treatment resulted in severely retarded growth of the *AvrPto* transgenic seedlings both in the darkness and under light (Supplementary Figs. S3 and S4). Unlike the *bak1* mutant, which displayed open cotyledons in the dark, the *AvrPto*-expressing seedlings did not show open cotyledons in the dark but were severely reduced in hypocotyl elongation, a phenotype that is also shared by the *brl1-5* mutant. In addition, root growth was severely arrested in the *AvrPto* seedlings. The *AvrPto* mature plants showed epinastic leaves and delayed flowering, which resembled *brl1* mutant phenotypes. Thus, it appears that transgenic expression of *AvrPto* leads to a pleiotropic phenotype indicative of both BR signaling defects and defects unrelated to BR signaling. Contrary to our observation, Shan and associates (2008) reported an open cotyledon phenotype in dexamethasone-inducible *AvrPto* transgenic seedlings, a difference possibly explained by the different expression systems used.

It was recently shown that several receptor-like cytoplasmic kinases (RLCK) called BR-signaling kinases (BSK) are substrates of BRI1 and mediate BR signaling (Tang et al. 2008). In addition, another RLCK, CDG1, was also proposed to function in BR signaling (Muto et al. 2004). The inability of AvrPto to interact with BAK1 prompted us to test if AvrPto can interact with BSK or CDG1. Co-IP assays showed that AvrPto can interact strongly with both BSK3 and CDG1 in protoplasts (Fig. 4). A weak interaction with BSK1 was also

detected. In contrast, BIK1, an RLCK acting downstream of FLS2, EFR, and CERK1 (Lu et al. 2010; Zhang et al. 2010), did not interact with AvrPto (Fig. 4). These results indicate that AvrPto can interact with protein kinases downstream of the BRI1-BAK1 receptor complex, providing an explanation for the observed BR phenotype in *AvrPto* plants. Pto and Fen belong to the receptor-like kinase (RLK) superfamily and share greatest homology with the CrRPK1L-1 subfamily kinases when aligned with *Arabidopsis* proteins (Supplementary Fig. 5). AvrPto is capable of interacting with CrRPK1L-1 member At2g23200 (Xiang et al. 2008), RLCK VII member CDG1, RLCK XII members BSK1 and BSK3, and leucine-rich repeat XII members FLS2 and EFR (Xiang et al. 2008). Thus, AvrPto does not appear to target a particular RLK subfamily. It is possible that its specificity is determined by the tertiary structure of the kinases.

AvrPto prevents the phosphorylation of BIK1 but not the ligand-induced FLS2-BAK1 association.

We previously proposed that AvrPto blocks PAMP signaling by inhibiting the kinase activity of PRR, including FLS2 and EFR (Xiang et al. 2008). We recently showed that BIK1 and its close homolog PBL1 are required for PTI signaling downstream of FLS2, EFR, and CERK1. Flg22 treatment leads to FLS2-dependent phosphorylation of BIK1 in protoplasts and in plants, suggesting that BIK1 is a substrate for FLS2. We also showed that transient expression of AvrPto in protoplasts prevents the flg22-induced phosphorylation on BIK1 (Zhang et al. 2010). To further determine if AvrPto blocks the ligand-induced phosphorylation of BIK1 in plants, we crossed an *AvrPto-FLAG* transgenic line with a *BIK1-HA* transgenic line. Figure 5 shows that, while the *BIK1-HA* plants lacking *AvrPto* showed a typical flg22-induced BIK1 phosphorylation, the expression of AvrPto abolished this phosphorylation. These results are consistent with a role of AvrPto as a kinase inhibitor.

It was reported that AvrPto impedes the flg22-induced FLS2-BAK1 association (Shan et al. 2008). We independently verified

Fig. 3. AvrPto interacts with FLS2 in plants. Eight-day-old seedlings with (+) or without (–) the *AvrPto-FLAG* transgene were induced with 10 μM estradiol for 2 days, and membrane protein was extracted for anti-FLAG immunoprecipitation (IP). The presence of FLS2 or BAK1 in the immune complex was determined using anti-FLS2 (eight experiments) or anti-BAK1 (two experiments) antibodies. Similar results were obtained in these experiments. IB = immunoblot.

Fig. 4. AvrPto can interact with BSK1, BSK3, and CDG1 but not with BIK1 in protoplasts. Hemagglutinin (HA)-tagged BSK1, BSK3, BIK1, and CDG1 were coexpressed with AvrPto-FLAG in Col-0 protoplasts, and total protein was immunoprecipitated with anti-AvrPto antibodies. The presence of AvrPto-FLAG and HA-tagged proteins in the immune complex was determined by immunoblot (IB), using appropriate antibodies. Asterisk indicates heavy chain of rabbit immunoglobulin G (IgG). Note that in the coimmunoprecipitation (co-IP) experiment, rabbit anti-AvrPto antibodies were used instead of mouse anti-FLAG antibody to avoid high background signal from mouse IgG heavy chain, which obstructs the observation of BSK, BIK1, and CDG1. The results shown are representative of three experiments.

Fig. 5. AvrPto prevents flg22-induced BIK1 phosphorylation in plants. *BIK1::BIK1-HA* transgenic plants (Zhang et al. 2010) of the indicated genetic background were sprayed with 10 μ M flg22 for 10 min, and mobility of BIK1-HA (hemagglutinin) in sodium dodecyl sulfate-polyacrylamide gel electrophoresis was examined with anti-HA immunoblot. The slower migration of BIK1-HA following flg22 treatment is indicative of phosphorylation (Zhang et al. 2010). The experiment was repeated three times with similar results.

this result by using co-IP assays in protoplasts. Figure 6A shows that flg22 induced a strong interaction between FLS2-FLAG and BAK1-HA either in the presence or absence of the nYFP-tagged AvrPto (Xiang et al. 2008). Likewise, FLS2-HA and BAK1-FLAG also showed flg22-induced association, regardless of the presence or absence of AvrPto-nYFP (Fig. 6B). To unequivocally determine if the PTI-inhibitory activity of AvrPto is correlated with the proposed interference of FLS2-BAK1 interaction, we cotransfected the same protoplasts with FLS2-FLAG, BAK1-HA, AvrPto-nYFP, and the FRK1-LUC reporter plasmids. The same protoplasts were assayed for flg22-induced reporter gene expression and FLS2-BAK1 interaction. The expression of AvrPto did not alter viability of the protoplasts but did reduce the *FRK1-LUC* expression to the background level, indicating that AvrPto completely abolished FLS2-mediated signaling (Fig. 6C). The interaction between FLS2-FLAG and BAK1-HA, however, was normal in the pres-

Fig. 6. AvrPto does not interfere with the flg22-induced FLS2-BAK1 interaction. **A**, Flg22 induces an interaction between FLS2-FLAG and BAK1-HA (hemagglutinin) in the presence of AvrPto-nYFP. **B**, Flg22 induces an interaction between FLS2-HA and BAK1-FLAG in the presence of AvrPto-nYFP. **C**, AvrPto-nYFP completely blocks flg22-induced *FRK1* expression in the presence of FLS2-BAK1 interaction. Col-0 protoplasts were transfected with the indicated constructs (A to C) and the FRK1-LUC reporter construct (C), were induced with 1 μ M flg22 for 10 min before protein was extracted for coimmunoprecipitation. A fraction of the protoplasts in C were examined for FRK1-LUC expression and protoplast viability (%) at 3 h. Error bars indicate standard deviation. The experiments were repeated three times with similar results.

ence of AvrPto. Thus, our experiments yielded no evidence for an effect of AvrPto on FLS2-BAK1 interaction.

DISCUSSION

In this study, we show that both the transiently expressed FLS2 and the endogenous FLS2 interact strongly with AvrPto. Our co-IP and BiFC assays on protoplasts always detected a strong AvrPto-FLS2 interaction but not an AvrPto-BAK1 interaction. We occasionally detected a weak BAK1 signal in the AvrPto immune complex only when both BAK1 and FLS2 were overexpressed in the same protoplasts. Furthermore, treatment of these protoplasts with flg22, which induces a strong FLS2-BAK1 interaction, enhanced the BAK1 protein abundance in the AvrPto immune complex. In contrast, the AvrPto-FLS2 interaction is independent of flg22 treatment (Xiang et al. 2008). These results are consistent with our previous findings that AvrPto directly interacts with FLS2 and indicate that the observed AvrPto-BAK1 co-IP occurred indirectly through a FLS2-BAK1 interaction.

Our co-IP assay results are in conflict with those of Shan and associates (2008), who reported a preferential interaction of AvrPto with BAK1. Although protoplast-based co-IP assays were performed in both studies, subtle differences in experimental procedures exist. Co-IP assays involve multiple steps of sample handling that could potentially introduce variations from different labs. For example, our co-IP assays involve eight washes of beads containing the immune complex (Xiang et al. 2008; this study), whereas five washes were done by Shan and associates (2008). In addition, our washing buffer contained higher concentrations of Triton X-100 than that of Shan and associates (2008). Different stringencies in the co-IP assays might have caused opposite results. To minimize potential artifacts from sample manipulation during the experiments, we performed BiFC assays, and our results clearly supported an interaction of AvrPto with FLS2 but not BAK1. Thus, our conclusion is supported by protoplast- and plant-based co-IP assays from different orientations and BiFC assays.

It was reported that the AvrPto^{S46P} mutant interacted with FLS2 but not BAK1 and that this mutant was unable to interfere with the flg22-induced FLS2-BAK1 interaction (Shan et al. 2008). The differential interaction was thought to support the biological significance of AvrPto-BAK1 interaction (Shan et al. 2008). However, this mutant is highly unstable and does not accumulate in *P. syringae* (Shan et al. 2000), a fact that complicates the interpretation of results.

Our results showed that accumulation of a high level of AvrPto in transgenic plants can lead to pleiotropic developmental phenotypes that may be explained, in part, by defects in the BR pathway. Because BAK1 plays a dual role in BR and PAMP signaling, it becomes a logical candidate of AvrPto target. However, our results clearly showed that AvrPto does not interact with BAK1. Instead, AvrPto can interact with other protein kinases required for plant development. For example, AvrPto can interact with the protein kinases BSK3 and CDG1, two RLCK that function in BR signaling (Muto et al. 2004; Tang et al. 2008), suggesting that overexpression of AvrPto in plants can potentially block BR signaling downstream of BRI1 and BAK1. This provides an alternative explanation for the observed BR signaling defects and suggests that developmental phenotypes of AvrPto-transgenic plants do not necessarily provide an accurate prediction of its physiological targets.

Shan and associates (2008) suggested that AvrPto physically impedes the flg22-induced FLS2-BAK1 association. However, our analyses failed to detect such an effect, although AvrPto completely blocked PTI signaling in the same protoplasts. We previously showed that AvrPto can act as a kinase inhibitor to

block FLS2 and EFR kinase activity, at least in vitro (Xiang et al. 2008). This is consistent with the finding that AvrPto binds Pto as a pseudosubstrate (Xing et al. 2007). AvrPto displayed significant kinase inhibitory activity when AvrPto and Pto are mixed at a ratio of approximately 1:1, and the kinase inhibition activity requires AvrPto tyrosine 89, which is essential for AvrPto-Pto and AvrPto-FLS2 interaction (Xiang et al. 2008; Xing et al. 2007). These results indicate that AvrPto inhibits protein kinases through a direct protein-protein interaction. We recently showed that the BIK1 directly interacts with FLS2, EFR, and CERK1 to mediate PTI. BIK1 is phosphorylated in vivo upon flg22 stimulation in a FLS2-dependent manner, suggesting that BIK1 is a substrate of FLS2. This phosphorylation is blocked by AvrPto in both protoplasts and plants. Because AvrPto does not interact with BIK1 in vivo, it is likely that AvrPto inhibits the kinase activity of FLS2 to block BIK1 phosphorylation.

Taken together, our extensive analyses on protein-protein interactions among AvrPto, FLS2, and BAK1 in previous reports and this study clearly indicate that FLS2 but not BAK1 is targeted by AvrPto. The FLS2-AvrPto interaction leads to a blockage of phosphorylation events downstream of FLS2.

MATERIALS AND METHODS

Plants.

Plants used in this study include the *bak1-4* (Chinchilla et al. 2007), *fls2* (Xiang et al. 2008), and *bri1-5* (Noguchi et al. 1999) mutants, *BIK1-HA* (Zhang et al. 2010) and estradiol-inducible *AvrPto-3xFLAG* transgenic lines (Xiang et al. 2008), wild-type Col-0, and WS. Plants were grown on soil in growth rooms at 23°C during the day and 20°C during the night, with a 10-h light photoperiod and 75% humidity. Alternatively, seedlings were grown on vertical phytoagar plates containing one-half Murashige Skoog (MS) medium, 1.5% sucrose, and 10 μ M estradiol (pH 5.7) in the dark or under continuous light. Soil-grown 5-week-old plants were used for protoplast preparation, and light-grown 8-day-old seedlings on plates were used for co-IP in plants.

Constructs.

The FLS2-HA, FLS2-cYFP, AvrPto-FLAG, AvrPto-nYFP, and BSK1-HA constructs have been reported previously (Xiang et al. 2008; Zhang et al. 2010). For the FLS2-FLAG and BAK1-HA constructs, the native *FLS2* and *BAK1* promoter and coding region were polymerase chain reaction (PCR)-amplified and were inserted into pUC19-35S-FLAG-RBS and pUC-35S-HA-RBS vector, respectively (Xiang et al. 2008). For the BAK1-FLAG construct, the *BAK1* cDNA was PCR-amplified and was inserted into pUC19-35S-FLAG-RBS vector. The *AvrPto* coding region was inserted into pUC-35S-HA-RBS, resulting in an AvrPto-HA construct. For the BAK1-cYFP construct, full-length *BAK1* was amplified from cDNA and was cloned into the SPYCE vector (Walter et al. 2004). For HA-tagged BSK3 and CDG1 constructs, full-length *BSK1* and *BSK3* were PCR-amplified from cDNA and *CDG1* from genomic DNA and were cloned into 35S-HA-PUC. All constructs were verified by sequencing. Primers used for cloning are listed in Supplementary Table S1.

Protoplast transfection, FRK1-LUC reporter assay, and viability assay.

Protoplast preparation and transfection were performed as described (Asai et al. 2002), with minor modifications. Leaves of 5-week-old plants were sliced into approximately 1-mm strips and were placed in 15 ml of enzyme solution containing 1% cellulose R10, 0.2% macerozyme R10, 0.4 M mannitol, 20

mM KCl, and 20 mM MES, pH 5.7, with gentle shaking (40 rpm) on a rotary shaker for 2 to 3 h. The speed was then increased to 80 rpm for 1 min to release the protoplasts. The protoplasts were filtered through a mesh (75 μ m) and were centrifuged in a 50-ml tube at $100 \times g$ for 2 min, and the supernatant was carefully removed. The protoplasts were subsequently washed in 10 to 20 ml of cold W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, and 2 mM morpholine-ethanesulfonic acid [MES], pH 5.7), were centrifuged at $100 \times g$ for 2 min, were resuspended in 20 ml W5, and were incubated on ice for 30 min. The protoplasts were then collected by centrifugation at $100 \times g$ for 2 min and were resuspended in a solution containing 0.4 M mannitol, 15 mM MgCl₂, and 4 mM MES, pH 5.7, to a final concentration of 2×10^5 cells/ml. For protoplast transfection, 10 μ g of plasmid DNA purified by caesium chloride ultracentrifugation was added into 200 μ l of protoplasts in a microfuge tube and was mixed briefly. A 220- μ l 40% polyethylene glycol (PEG) 4,000 solution was then added and thoroughly mixed. The protoplasts were incubated at 23°C for 6 min, were diluted with 0.8 ml of W5 solution, and were mixed and centrifuged at $100 \times g$ for 2 min to remove PEG. The protoplasts were then resuspended gently, were diluted in 1 ml of W5, and were incubated overnight under the light. For the *FRK1-LUC* reporter assay, protoplasts were treated with 1 μ M flg22 for 3 h before *FRK1-LUC* activity was measured as described (Xiang et al. 2008). For the viability assay, a protoplast suspension (10^5 cells/ml) was mixed at a 1:1 ratio with 0.4% trypan blue solution, was loaded into the counting chambers of a hemocytometer, and was allowed to settle for 1 to 2 min before the ratio of stained to unstained cells was calculated.

Co-IP assays.

For co-IP in plants, 8-day-old light-grown seedlings were treated with 10 μ M estradiol on MS plates for 2 days, and total protein was isolated by homogenizing tissues with a buffer containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 300 mM sucrose, 1 mM EDTA, 5 mM NaF, 1 mM Na₃VO₄, and 1 $\times$ proteinase inhibitor cocktail (Roche Molecular Systems, Inc., Mannheim, Germany). The total protein was then centrifuged at $100,000 \times g$ for 1 h to isolate microsomes. Membrane protein was isolated by dissolving the microsome in a buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM NaF, 1 mM Na₃VO₄, and 1 $\times$ proteinase inhibitor cocktail (Roche). For protoplast-based co-IP, total protein was extracted from protoplasts with a buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM NaF, 1 mM Na₃VO₄, and 1 $\times$ proteinase inhibitor cocktail (Roche). For anti-FLAG IP, approximately 2 mg of protein was incubated with 10 μ l of agarose-conjugated anti-FLAG monoclonal antibody (Sigma, St. Louis) for 4 h at 4°C on a 360-degree rotary shaker. The beads were then collected and washed eight times with washing buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Triton X-100; 1 ml per wash). The bound protein was eluted with 100 μ l of elution buffer (5 mM 3xFLAG peptide, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4) overnight at 4°C. For anti-HA IP, approximately 2 mg of total protein was precleared with Protein-A-Agarose (Upstate Chemicon, Temecula, CA, U.S.A.) for 1 h, and the protein extract was then precipitated with 2 μ g of anti-HA antibody and Protein-A-Agarose for 4 h. Bound protein was eluted from beads by boiling in protein sample buffer. For anti-AvrPto IP, ~2 mg total protein was pre-cleared with Protein-A-Agarose (Upstate) for 1 h, and the protein extract was then precipitated with 2 μ g anti-AvrPto antibody and Protein-A-Agarose for 4 hrs. Bound protein was eluted from beads by boiling in protein sample buffer. One third of the im-

munoprecipitated protein was subjected to immunoblot analyses with anti-HA monoclonal antibody (TianGen, Beijing), anti-FLAG monoclonal antibody (Sigma), anti-FLS2 antibodies (Zhang et al. 2010), and anti-BAK1 antibody (Gao et al. 2009). Approximately 200 μ g of total protein was loaded as input control.

BiFC assays.

Arabidopsis protoplasts were cotransfected with cYFP-tagged full-length FLS2 or BAK1 and nYFP-tagged AvrPto or empty SPYNE vector. Complementation of YFP was visualized with a confocal microscope.

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